Prevention of Gamma Radiation-Induced Mortality in Mice by the Isoflavone Genistein

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ABSTRACT

There is a need to develop medical countermeasures to protect first-responders and remediation workers from the biomedical effects of ionizing radiation. Agents that can protect against ionizing radiation and that can be administered before radiation would permit rescue workers to perform needed operations. The ideal radioprotector would be nontoxic and would not degrade performance. Soy phytoestrogens have strong antioxidant activity and have been reported to have many beneficial health effects, including a reduction in bone loss and the incidence of some types of cancer. The most plentiful isoflavone from soybeans is genistein (4', 5, 7-trihydroxy-flavone). In the present study, the radioprotective and behavioral effects of an acute administration of the isoflavone genistein were investigated in adult CD2F1 male mice. They were administered a single subcutaneous dose of genistein, either 24 hr or 1 hr before a lethal dose of gamma radiation (9.5 Gy cobalt-60 at 0.6 Gy/min). The mice received vehicle or genistein at 3.12, 6.25, 12.5, 25, 50, 100, 200, or 400 mg/kg. For mice treated 24 hr before irradiation, there was a significant increase in 30-day survival for animals receiving genistein doses of 25 to 400 mg/kg (p < 0.001) compared to the vehicle control group. In contrast, the 30-day survival rates of mice treated with genistein 1 hr before irradiation were not significantly different from those of the vehicle control group. In a separate experiment, the toxicity of genistein was evaluated in non-irradiated male mice administered a single subcutaneous injection of vehicle, or genistein at 100, 200, or 400 mg/kg. Compared with controls at these genistein doses, there were no adverse effects on locomotor activity, grip strength, body weight, testes weight, or histopathology. The results demonstrate that a single subcutaneous administration of the flavonoid genistein at nontoxic doses provides protection against acute radiation injury. Future studies are planned to examine the mechanism for genistein's radioprotective effects. These studies will use human ex vivo whole-blood and murine in vivo spleen models and evaluate the effects of genistein on signal transduction involving DNA repair, apoptosis, and cytokine targets.

1.0   INTRODUCTION

Radioprotective agents are compounds that are administered before exposure to ionizing radiation to reduce its damaging effects, including radiation-induced lethality [Stone 2004]. They have applications in radiological terrorism, military scenarios, clinical oncology, space travel, radiation site cleanup [Johnson 2004, Mettler 2002, Nair 2001, Waselenko 2004]. Recently, the U.S. Office of Science and Technology Policy and the

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Homeland Security Council have made the development of new radioprotectors a top research priority [Pellmar 2005]. Although synthetic radioprotectors such as the aminothiols have yielded the highest protective factors, typically they are more toxic [Rades 2004] than naturally occurring protectors [Weiss 2003]. In general, the best radioprotective agents also have been reported to result in the highest behavioral toxicity [Landauer 1992, Landauer 2001a]. In a military radiation scenario, the effective mitigation of radiation-induced health consequences and performance-degrading effects can reduce the casualty load at medical treatment facilities, sustain a more effective operational force after a radiation exposure event, allow commanders to conduct operations in radiation field environments with reduced risk of decremented performance due to acute tissue injury, and reduce the negative psychological impact on personnel tasked to operate in contaminated environments. The ideal radioprotectant would be nontoxic, would not degrade performance, and would be effective after a single administration, particularly when expedited entry is required into an area with potential external radiation hazards.

Naturally occurring dietary components offer opportunities for development as effective chemopreventive and radioprotective agents because of their potential low toxicity [Coleman 2004, Sarkar 2004, Weiss 2003] Genistein (4',5,7-trihydroxyflavone), a naturally occurring isoflavone found in soybeans, has gained increasing attention because of its association with beneficial effects for treatment of cardiovascular disease, high blood pressure, osteoporosis, breast cancer, and prostate cancer [McCue 2004, Valachovicova 2004]. Its molecular formula is $C_{15}H_{10}O_5$ and its molecular weight is 270.24 daltons (Figure 1). The antioxidant and phytoestrogenic properties of genistein make it an excellent candidate for investigation as a radioprotectant.
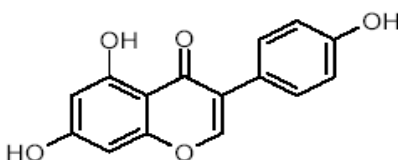

Figure 1: Structure of genistein

2.0   MATERIALS AND METHODS

2.1   Animals

Male CD2F1 mice (Harlan Laboratories, Indianapolis, IN) weighing 24 to 30 g were used in these studies. Mice were housed in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Animal rooms were maintained at $21° \pm 2°C$ with $50\% \pm 10\%$ humidity on a 12-hr light/dark cycle. Commercial rodent ration (Harlan Teklad Rodent Diet 8604) was available freely as was acidified (pH=2.5) water to control opportunistic infections [McPherson 1963]. All animal-handling procedures were performed in compliance with guidelines from the National Research Council (1996) and were approved by the Institutional Animal Care and Use Committee of the Armed Forces Radiobiology Research Institute (AFRRI).

2.2   Isoflavone Preparation

Genistein was solubilized in polyethylene glycol with a molecular weight of 400 (PEG-400) on the day of the experiment by 20 sec of sonication (Heat Systems-Ultrasonics Inc., Plainview, NY). Genistein and PEG-400 (PEG) were obtained from Sigma Chemical Company (St. Louis, MO). Saline, used as a control excipient,

was obtained from Abbott Laboratories (North Chicago, IL). All drugs were administered subcutaneously in a volume of 0.1 ml. The day of injection was considered day 0.

2.3   Experimental Design

Separate groups of mice were used to assess the acute toxicity of genistein in non-irradiated mice. Once an acceptable nontoxic dose of genistein was determined, radioprotection studies were performed.

2.4   Assessment of Acute Toxicity of Genistein in Non-Irradiated Mice

Acute toxicity experiments consisted of injecting mice with a single dose of genistein and observing them for clinical signs and behavioral toxicity over a 14-day period. After 14 days the animals were evaluated for any changes in pathology. Behavioral experiments were conducted in five groups of nonirradiated mice (N = 10/group). Mice were assessed for locomotor activity and forelimb grip strength. Each mouse, by group, received a single subcutaneous injection of saline, vehicle, or genistein at 100, 200, or 400 mg/kg of body weight. Behavioral assessments were made on days 1, 4, and 14 following injection. Behavioral tests measuring locomotor activity and grip strength have been recommended for the evaluation of chemical compounds by such agencies as the U.S. Food and Drug Administration (FDA) and the World Health Organization.

Locomotor activity was quantified by use of an automated computerized activity monitoring system (Omnitech Electronics, Columbus, OH) as previously described [Landauer 2001a]. Each monitor used an array of infrared photodetectors spaced 2.5 cm apart to determine the total distance traveled. Activity was monitored continuously for 12 hr during the dark portion of the light/dark cycle. Food and water were available throughout the testing period.

Forelimb grip strength performance was assessed during the light portion of the light/dark cycle using a procedure previously reported [Landauer 2001b]. Briefly, peak forelimb grip strength was measured in kilograms by a Chatillon Digital Force Gauge (Model DFI2, Greensboro, NC). The gauge was attached to a stainless steel T-bar. A mouse was placed with its forepaws on the T-bar and gently pulled backward by the tail at a steady rate until its grip was broken. To eliminate bias, the individual administering the grip strength test was unaware of the treatment received by the animal. Two trials per mouse were conducted and the average of these trials was computed to estimate forelimb grip strength.

The same nonirradiated animals used for the behavioral experiments were weighed throughout the 14-day period following injection. Clinical signs such as lethargy, fur condition, and general well-being were monitored at the time of weighing. Fourteen days after subcutaneous administration of genistein, nonirradiated mice from the two control groups (saline and vehicle) and those from the high-dose (400-mg/kg) genistein group were euthanized and necropsied. Each animal received a gross examination. In addition, tissues from the testes, liver, adrenal gland, mesenteric lymph node, spleen, and bone marrow of the femur and sternum were collected, fixed in buffered formalin, paraffin embedded, sectioned, and stained by hematoxylin and eosin. The wet weight of both testes without epididymides was determined before fixing in formalin. A board-certified veterinary pathologist examined all tissues.

2.5   Radioprotection Experiments

Mice were irradiated in a bilateral gamma radiation field at the Armed Forces Radiobiology Research Institute's (AFRRI) cobalt-60 facility. The midline tissue dose to the animals was varied as a function of the experiment and will be at a dose rate of 0.6 Gy/min. Control animals were sham irradiated. The alanine/ESR

(electron spin resonance) dosimetry system (American Society for Testing and Materials Standard E 1607) was used to measure dose rates (to water) in the cores of acrylic mouse phantoms. Phantoms were 3 inches in length and 1 inch in diameter, to simulate a mouse, and were located in every other compartment of the exposure rack. The ESR signals were measured with a calibration curve based on the standard calibration dosimeters provided by the National Institute of Standards and Technology (NIST). The overall uncertainty in the doses given to the calibration dosimeters at NIST was approximately 1.8% at 2σ. The accuracy of the calibration curve was verified by parallel measurements of doses to selected dosimeters at AFRRI and at NIST. The only corrections applied to the dose rates in phantoms were for the decay of cobalt-60 and for the small difference in the mass energy-absorption coefficients for water and soft tissue.

For the initial radioprotection studies, mice received a single subcutaneous injection of genistein before 9.5-Gy gamma irradiation at a dose rate of 0.6 Gy/min. This is the approximate $LD_{95/30}$ (the radiation dose that is lethal to 95% of the animals within 30 days) for the CD2F1 male mouse. Mice were each administered a single subcutaneous injection of saline, vehicle, or genistein at 3.125, 6.25, 12.5, 25, 50, 100, 200, or 400 mg/kg either 24 hr (N = 16-48/group) or 1 hr (N = 16-32/group) before irradiation and were monitored for 30 days.

The optimal administration time and protective genistein dose were selected for assessment of the dose-reduction factor (DRF). For determination of the DRF, mice in the saline groups were irradiated with doses in the range of 7.5 to 9.5 Gy; for the vehicle groups, 8 to 10 Gy; and for the genistein groups, 9.5 to 11 Gy. To determine the protective ratio of genistein against acute, lethal gamma irradiation, the DRF was calculated by dividing the $LD_{50/30}$ of the genistein-treated animals by the $LD_{50/30}$ of the vehicle-treated animals. The DRF also was calculated for the drug vehicle by using the $LD_{50/30}$ values for PEG-400 and saline-treated animals.

2.5   Statistical Analysis

An analysis of variance was used to statistically analyze locomotor activity, grip strength, body weight, and testes weight data. The Fisher's exact test was used for analysis of survival data. The $LD_{50/30}$ was determined by probit analysis.

3.0   RESULTS

3.1   Acute Toxicity Assessment

No toxicity was observed. When non-irradiated mice were tested on days 1, 4, and 14 after injection, genistein doses of 100 to 400 mg/kg did not alter locomotor activity or grip strength, compared with the results for the vehicle or saline control group (Figure 2). Results for the PEG-400 vehicle group did not differ from those for the saline control group. The data for the locomotor activity test are presented as the sum of the 12-hr activity-recording period (Figure 2). While there were no significant differences among groups, the activity of all groups was lower on days 4 and 14, compared with the activity on day 1.
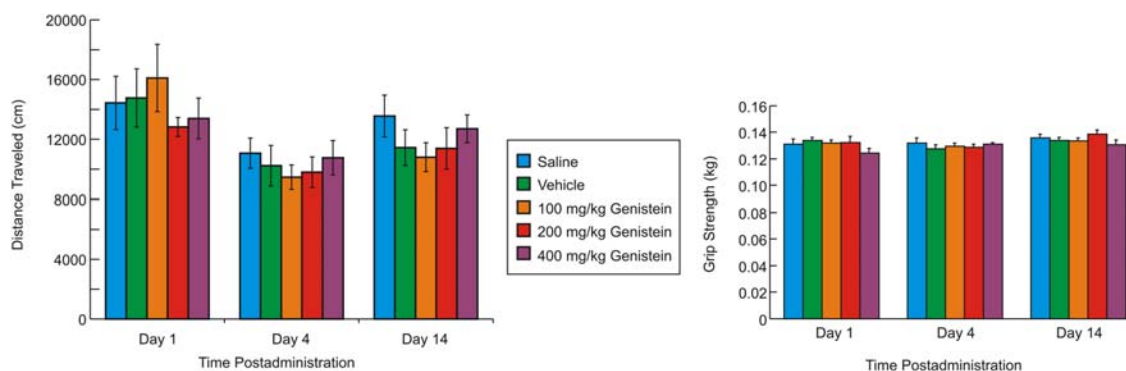
Figure 2: Effect of genistein on locomotor activity (left) and fore-limb grip strength (right). Mice were evaluated on days 1, 4 and 14 after acute subcutaneous administration of saline, PEG vehicle, or 100, 200, or 400 mg/kg of genistein. There were no significant differences among groups for either behavioral measure.

Following an acute subcutaneous administration of genistein at 100, 200, or 400 mg/kg, there were no treatment-related effects on body weight (Figure 3). No adverse clinical signs were observed. In addition, there were no treatment-related effects observed upon gross necropsy. Tissue sections of liver, adrenal gland, mesenteric lymph node, spleen, testes, and bone marrow of the femur and sternum had no significant histological changes associated with treatment. There were no treatment effects on testes weight (Figure 4).
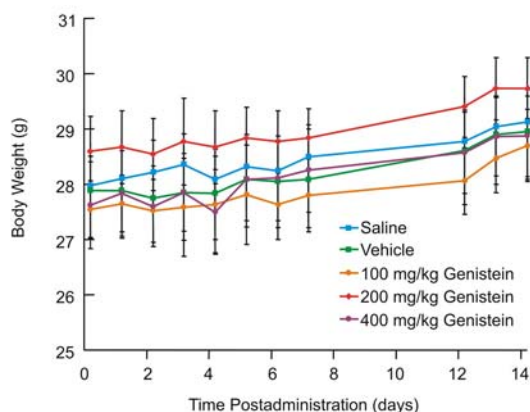

Figure 3: Mean ± SEM body weight of mice administered an acute subcutaneous dose of saline, PEG vehicle, or 100, 200, or 400 mg/kg of genistein. There were no significant differences among groups.
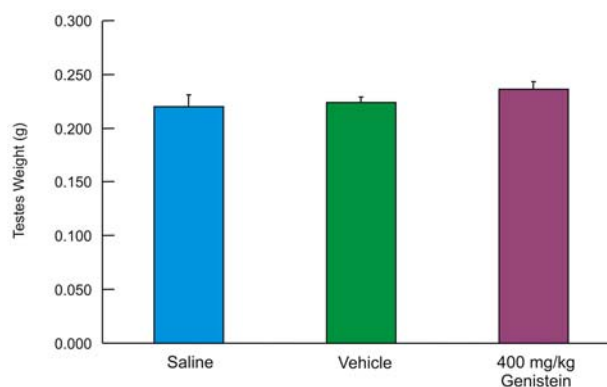
Figure 4: The effect of a single subcutaneous injection of saline, PEG vehicle, or 400 mg/kg of genistein on testes weights. Weights reflect the sum, 14 days after injection, of both testes without epididymes removed. Vertical lines represent the mean ± SEM. There were no significant differences among groups.

3.2   Radioprotection Experiments

When genistein was administered 24 hr before irradiation, there was a significant (p < 0.001) enhancement in 30-day survival for groups of mice that received genistein doses of 25 to 400 (Figure 5). The optimal radio-protective genistein dose of 200 mg/kg afforded significantly (p < 0.01) more protection than the lower doses (25-100 mg/kg); however, the level of protection was not significantly different from that for the genistein dose of 400 mg/kg. The survival curve illustrates that, compared with the control group data, the time to death is (p <0.05) shifted significantly to the right for mice treated with 25-400 mg/kg genistein 24 hr before irradiation (Figure 5). In contrast, when mice were administered genistein 1 hr before exposure to gamma radiation, doses ranging from 3.125 to 400 mg/kg failed to increase 30-day survival (Figure 6). There was also no significant effect on the time to death.
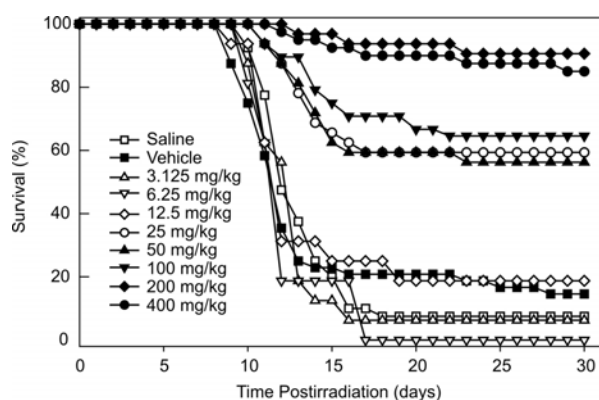

Figure 5: Survival curves for mice treated with a single subcutaneous injection of saline, PEG vehicle, 3.125-400 mg/kg genistein 24 hr before 9.5-Gy cobalt-60 gamma irradiation. Doses of 25 mg/kg and above were significantly different from controls.
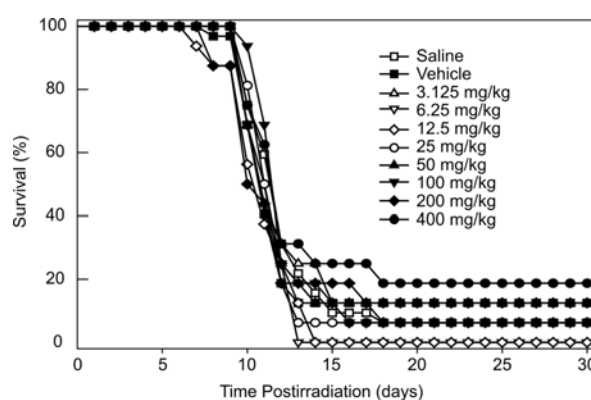
Figure 6: Survival curves for mice treated with a single subcutaneous injection of saline, PEG vehicle, or genistein, 1 hr before 9.5-Gy cobalt-60 gamma irradiation. No radioprotection was observed.

The genistein dose of 200 mg/kg, which resulted in the highest survival rate when administered to mice 24 hr before irradiation, was used to determine the DRF (Figure 7). The $LD_{50/30}$ (95% confidence intervals) for saline was 8.68 Gy (8.52, 8.85), for PEG-400 vehicle, 9.04 Gy (8.91, 9.17), and for 200 mg/kg genistein, 10.50 Gy (10.40, 10.58). This resulted in a DRF of 1.16 for 200-mg/kg genistein, when compared with vehicle (p < 0.001), and a DRF of 1.04 for PEG-400 when compared with saline (p < 0.003).

4.0   DISCUSSION

The results of this study demonstrate that, in mice, a single subcutaneous dose of the isoflavone genistein mitigates the lethal effects of radiation exposure without changes in behavior, body weight or histopathology. Doses of 25 to 400 mg/kg genistein were radioprotective when administered 24 hr before irradiation, but were not protective when given 1 hr before irradiation.
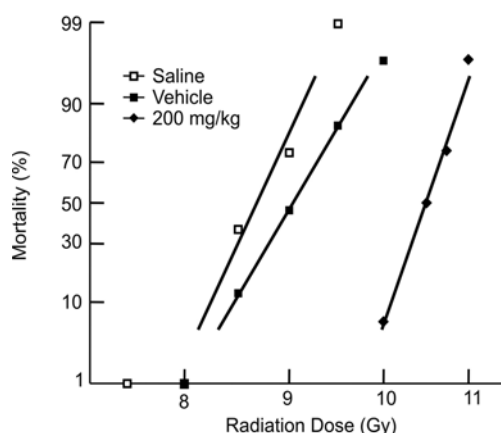
Figure 7: Probit mortality curves of mice exposed to whole-body cobalt-60 gamma radiation. Mice were administered, 24 hr before irradiation, a single subcutaneous injection of saline, PEG-400 vehicle, or 200 mg/kg genistein. The DRF for genistein (1.16) was calculated from the ratio of the LD_{50} at 30 days for genistein-treated to vehicle-treated mice (p < 0.001). The DRF for vehicle, compared with that for saline, was 1.04 (p < 0.003; N = 16-32 mice/group).

Radioprotection without significant decrement in performance is essential for the selection of protective agents to be used in military applications, emergency response personnel, or manned space flight. Genistein afforded protection without behavioral disruption. The behavioral tests in the present study revealed that an acute subcutaneous injection of genistein at doses as high as 400 mg/kg did not result in behavioral alterations over the 14-day evaluation period. The decrease in motor activity, which we observed across all groups after the initial testing on day 1, commonly is observed when repeated locomotor tests are conducted, and results from behavioral habituation.

A single subcutaneous administration of genistein up to 400 mg/kg caused no behavioral toxicity and no gross or microscopic pathological changes compared with the results for the control groups. In addition, no adverse changes in body weight were observed. Similarly, no toxic effects were reported in mice after a single intra-peritoneal genistein injection of 100 mg/kg [Asahi 1981] or 500 mg/kg [Ogawara 1986].

A DRF of 1.16 (based on 30-day survival) was obtained for male mice administered a single genistein injection of 200-mg/kg 24 hr before gamma irradiation. Although this may be a modest increase in survival, it is a statistically significant increase and occurs in the absence of any toxicity or performance decrement. Mice administered the PEG-400 vehicle also exhibited a significant enhancement in 30-day survival, with a DRF of 1.04. PEG-400 previously was shown to be radioprotective [Shaeffer 1986], as were other excipients such as Emulphor [Landauer 2001a] and dimethyl sulfoxide [Moos 1967].

While our data demonstrate that genistein protects normal tissues from ionizing radiation, this isoflavone has been reported not to protect tumors, and can enhance the efficacy of both radiotherapy and chemotherapy treatments. For example, genistein improved radiotherapy of prostate tumors *in vivo* [Hillman 2004], chemo-therapy of breast cancer cells [Satoh 2003] and chemo-radiotherapy of lung carcinoma cells [McDonnell 2004].

Although we did not measure genistein plasma levels in this study, it is not likely that protection correlates

with these levels because genistein is eliminated rapidly from systemic circulation in mice. When a genistein dose of 25 mg/kg was administered parenterally to CD2F1 male mice, plasma levels decreased from 30.8 μg/ml at 3 min postinjection to 0.036 μg/ml after 2.5 hr postinjection [Supko 1995]. Therefore, the parent compound, genistein, is unlikely to be present in the plasma at the time of irradiation in mice treated 24 hr before exposure; however, a metabolite could be responsible for the radioprotection. Additionally, genistein was not radioprotective when administered subcutaneously 1 hr before irradiation but was protective when given 24 hr before irradiation.

Genistein possesses a number of biological properties that may contribute to its radioprotective efficacy. These include its estrogenic activity, antioxidant properties, immunostimulatory activity as well as its involvement in signal transduction pathways where it is an inhibitor of topoisomerase, protein kinase, and caspases involved in apoptotic pathways [Erlejman 2004, Polkowski 2000, Wang 2000]. These properties have been associated previously with radioprotection [Thompson 1965, Uckun 1992, Weiss 1990, Weiss 2000]. Because we observed radioprotection when genistein was administered 24 hr but not 1 hr before irradiation, an indirect mechanism for radioprotection, such as cytokine release, or DNA repair also is possible. In a pilot study, we observed that in an *ex vivo* blood model [Grace 2002], human blood that was incubated with genistein for 16 hr and subsequently irradiated with 2 Gy, exhibited a dose-dependent increase in the expression of the DNA repair gene, GADD45a.

Because genistein is derived from a natural food substance, we have also investigated radioprotection by oral administration in mice. In preliminary studies, animals that received a single oral gavage of genistein (400 mg/kg) either 1 hr or 24 hr before 9.5-Gy irradiation did not show radioprotection. However, when repeated daily doses of genistein (100 mg/kg) were given for 4 days before and 4 days after irradiation, we observed a significant increase in survival of Genistein-treated mice compared to vehicle control animals. Future studies are planned to address radioprotection by oral delivery.

Preclinical research demonstrated that genistein has an excellent safety profile and is suitable for adult human clinical trials [Faqi 2004, Landauer 2003]. Based on differences in surface area and the more rapid metabolism of isoflavones in mice, a conversion factor of 12 (Freireich *et al.* 1966) can be used to estimate the human equivalent dose from the mouse dose. With this allometric scaling figure, doses of 100 and 200 mg/kg genistein in the mouse would be equal to approximately 8 and 16 mg/kg respectively, in humans. Clinical trials have used oral doses as high as 16-mg/kg body weight of genistein in humans. These doses were reported to be well tolerated with no clinically related drug toxicity [Bergan 2001]. Consequently, the experimental doses used in the mouse experiments appear to be clinically feasible.

In summary, our research demonstrates that genistein is an effective nontoxic radiation protective agent against radiation-induced lethality in mice. Plans for further development of this compound have been initiated by collaboration with industrial partners.

ACKNOWLEDGMENTS

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